Eur Food Res Technol (2001) 212:566–569
DOI 10.1007/s002170100296

Reprint #8569

ORIGINAL PAPER

Jerry W. King · Janet M. Snyder · Hans Frykman
Angela Neese

Sterol ester production using lipase-catalyzed reactions in supercritical carbon dioxide

Received: 26 September 2000 / Revised version: 23 November 2000 / Published online: 29 March 2001

Abstract Synthesis of sterol and stanol esters is of importance, due to their recent recognition and application in the food and nutriceutical industries as cholesterol-lowering agents. In this study, several enzymes were evaluated to determine the best catalyst and optimal conditions for the reaction between various fatty acids and cholesterol or sitostanol in supercritical carbon dioxide ($SC-CO_2$). Using an analytical supercritical fluid extraction (SFE) unit, the lipase derived from *Burkholderia cepacia*, Chirazyme L-1, was determined to be the most selective for facilitating the desired reactions. Fatty acids C_8–C_{18}, pressures between 20.7 MPa and 31 MPa, a temperature range of 40–60 °C, along with variable flow rates, and initial static hold times were used to evaluated the feasibility of the above reaction. The yield of the cholesterol esters, as measured by supercritical fluid chromatography (SFC), ranged from 90% for caprylic acid to 99% for palmitic acid, while the corresponding reaction between sitostanol and the same fatty acids produced yields of 92% for caprylic acid and 99% for palmitic acid, respectively. The extraction apparatus was modified to provide a continuous flow of the reagent fatty acid and sterol/stanol over the enzyme bed, thereby allowing continuous production of the desired esters which averaged a 99% yield under optimal conditions.

Keywords Lipase · Reaction · Sterol ester · Supercritical carbon dioxide

J.W. King (✉) · J.M. Snyder · A. Neese
Food Quality & Safety Research,
National Center for Agricultural Utilization Research,
Agricultural Research Service/USDA,
1815 N. University Street, Peoria, Illinois 61604 USA
e-mail: kingjw@mail.ncaur.usda.gov

H. Frykman
New Crops Research,
National Center for Agricultural Utilization Research,
Agricultural Research Service/USDA,
1815 N. University Street, Peoria, Illinois 61604 USA

Introduction

The utilization of supercritical carbon dioxide ($SC-CO_2$) as a reaction medium confers many advantages, among which are an environmentally-compatibility, zero chemical residue in the synthesized product, and considerable processing flexibility [1]. When an $SC-CO_2$-based synthesis is coupled with the use of a catalyst, such as lipase [2], an all-natural process results that is particularly applicable to producing additives that can be incorporated directly into food formulations.

Lipase-catalyzed reactions in $SC-CO_2$ have been reported by numerous investigators [3, 4, 5] and several excellent reviews summarize activity in this area [6, 7]. Previously we have reported synthesis to make simple esters [8], conducted transesterifications to make methyl esters [9], patented a glycerolysis process [10], and performed randomization of fats/oils [11] in $SC-CO_2$, using a lipase derived from *Candida antarctica* [12], commercially known as Novozym 435. High quantitative yields when performing such transesterifications to make methyl esters have permitted application of the $SC-CO_2$/lipase reaction as an analytical method for quantitating fat levels in food products which are required under new food nutritional labeling guidelines [13, 14]. Recently, a Novozym 435-catalyzed transesterification has been utilized by one of the investigators as the initial step in a two-stage synthesis conducted under critical fluid conditions to produce fatty alcohols directly from vegetable oils [15].

In this study, the application of an $SC-CO_2$/lipase-based reaction has been used to synthesize sterol esters which have utility as functional food ingredients, namely as cholesterol-lowering agents as reported in the literature [16]. The success of utilizing chemically-modified tall oil-derived sterols in margarine spreads and other food products for their cholesterol-lowering propensity suggests that natural synthetic routes for making such food additives from alternative agricultural sources would be welcomed. In the case of the tall oil-derived additives, naturally derived sterols are hydrogenated to stanols, and then reacted to form esters to enhance their compatibility with the oils constituting margarine spreads [17, 18].

The feasibility of conducting lipase-catalyzed reactions between sterols/stanols and various *n*-alkanoic acids was surveyed in this study. Specifically, several lipases have been evaluated with respect to their ability to form long chain esters with a model sterol and stanol, respectively, under SC-CO_2 conditions. Syntheses were conducted under both static and dynamic flow conditions using a micro reactor approach to expedite experimentation and minimize the expense of costly reagents. Reaction yields were optimized with respect to pressure, temperature, and flow rates.

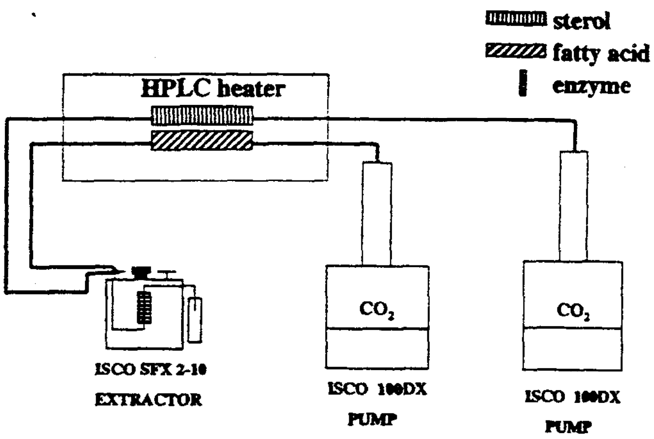

Fig. 1 Continuous reaction system for synthesis of sterol/stanol esters in SC-CO_2

Materials and methods

Materials. Candidate lipases were obtained from commercial sources as reported previously [19] while the Chirazyme L-1, derived from *Burkholderia cepacia* was obtained from the Boehringer Mannheim Corporation (Indianapolis, IN, USA). The reagent fatty acids *n*-octanoic, decanoic, dodecanoic (lauric), hexadecanoic (palmitic), and octadecanoic (stearic) were all purchased from Aldrich Chemical Company (Milwaukee, WI, USA), as was the cholesterol used in the study. Sitostanol was obtained from TCI America (Portland, OR, USA).

Apparatus. Initial semi-continuous reactions were conducted using an Isco Model SFX 2–10 supercritical fluid extraction (SFE) system available from Isco, Inc (Lincoln, NE, USA) and Isco Model 100DX syringe pumps for delivery of CO_2. For reactions conducted in the continuous flow mode, a modified SFX 2–10 unit was utilized as shown in Fig. 1. Here the two Isco Model 100DX syringe pumps were typically filled with CO_2 at a pressure of 27.6 MPa. Two empty columns were then added to the exit lines of both syringe pumps to serve as reagent reservoirs for the sterol/stanol or fatty acids, respectively, and inserted into an HPLC column temperature control module (Model 125–0425, Bio-Rad Corporation, Hercules, CA, USA). Feed lines from these reagent reservoirs were then merged in a tee prior to entering the entry port to the Isco Model SFX 2–10 extractor. A 1-ml/min heated restrictor was utilized at the exit port of the Model SFX 2–10 module to control the overall flow rate.

Experimental procedure. The batch, semi-continuous reactions were conducted using a 2.5-ml cell in the Isco SFX 2–10 unit as a reaction chamber. Typically about 750 mg of supported enzyme was initially placed in the cell, followed by a glass wool plug, and then a mixture containing 20 mg of sterol or stanol and 40 mg of fatty acid. The cell was then inserted into the heated oven of the Model SFX 2–10 so that the SC-CO_2 initially flowed over the reactants thereby solubilizing them in the SC-CO_2 before passing over the supported enzyme bed. After conversion over the enzyme bed, the reaction products and excess reactants dissolved in the SC-CO_2 underwent decompression through the heated restrictor prior to collection in a vial. While conducting reactions in this mode, the pressure was varied from 20.7 MPa to 31 MPa, the temperature from 40 °C to 60 °C. Carbon dioxide flow rate was also varied by changing the size of the heated restrictor, so that the flow rate varied from 0.5 ml/min to 2.0 ml/min. These flow rates are for CO_2 at the conditions inside the reaction cell, i.e., under the specified pressure and temperature. Initial static hold times (before commencing the flow of CO_2) were also varied from 0 min to 15 min.

The continuous flow reaction was conducted on the modified SFX 2–10 unit described above whose extraction cell was filled with about 0.8 g of Chirazyme L-1 enzyme. SC-CO_2 was delivered from the two Isco 100DX syringe pumps at a pressure of 27.5 MPa. The sterol component (0.4 g) was added to the reagent reservoir placed in-line after Pump A, while the fatty acid (0.4 g) was inserted into the reservoir in-line after Pump B. The cell temperature of the Model SFX 2–10 unit and HPLC heater module were both set at 50 °C. Flow rates from each Model 100DX pump was varied to optimize production of the sterol ester.

Analytical procedure. The results of the enzymatic reaction were monitored using a Dionex Model 600 supercritical fluid chromatograph (Dionex Inc., Salt Lake City, UT, USA). A Dionex SB-Octyl 50 column (10 m×100 μm×0.5 μ film thickness) was used to separate the products and reactants. A pressure gradient program consisting of a 5-min initial hold at 10.1 MPa followed by a linear pressure ramp of 0.5 MPa/min to 15.2 MPa, followed by a pressure ramp from 15.2 MPa to 28.4 MPa at 0.2 MPa/min, and finally an increase in pressure from 18.2 MPa to 28.4 MPa at 0.5 MPa/min was used to separate the reaction products. The column temperature was held isothermally at 100 °C during the pressure ramping program Samples were injected onto the column using a Valco injection valve (Valco Inc., Houston, TX, USA) equipped with a 200-nl injection loop utilizing a time-split injection mode of 1.8 s. Detection was accomplished using a flame ionization detector (FID) held at 350 °C.

Results and discussion

Four enzyme candidates: Novozyme 435, Chirazyme L-1, Chirazyme L-3, and Lypozyme IM, were initially screened using the batch, semi-continuous method described in the materials and methods section. Cholesterol was chosen as the model sterol for these reactions and others based on its availability and low cost relative to sitostanol. Chirazyme L-1 was found to be the optimal lipase based on high overall yields for the ester formed between lauric or palmitic acid with cholesterol. This enzyme was than used in the remainder of the esterification reactions.

Figure 2 shows the effect of extraction/reaction pressure on the yield for the palmitic acid ester of cholesterol. At 27.6 MPa, a maximum yield of almost 60% was obtained at a temperature of 50 °C, CO_2 flow rate of 2.0 ml/min, and an initial static hold time of 5 min before starting the CO_2 flow continuously. Use of the ini-

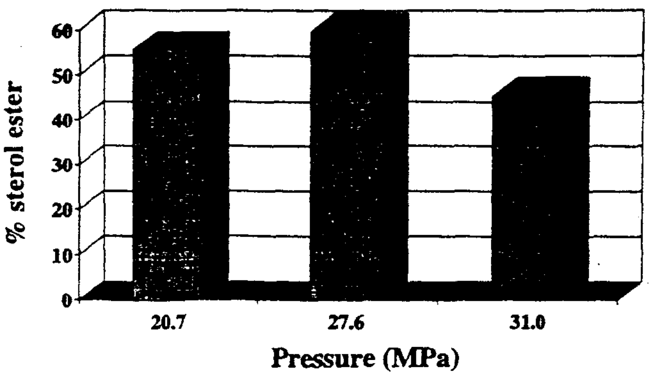

Fig. 2 Effect of pressure on cholesterol palmitate yield. Conditions: 50 °C, flow rate=2.0 ml/min, static hold time=2 min

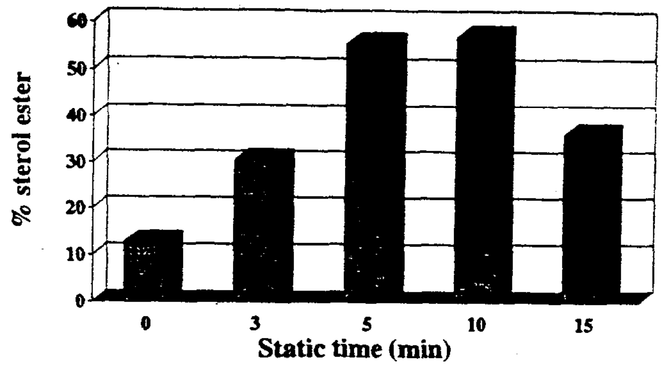

Fig. 4 Effect of static hold time on cholesterol palmitate yield. Conditions: 20.7 MPa, 40 °C, flow rate=2.0 ml/min

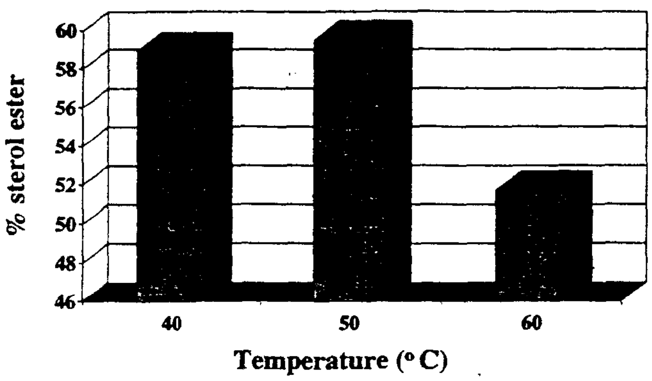

Fig. 3 Effect of temperature on cholesterol palmitate yield. Conditions: 27.6 MPa, flow rate=2.0 ml/min, static hold time=3 min

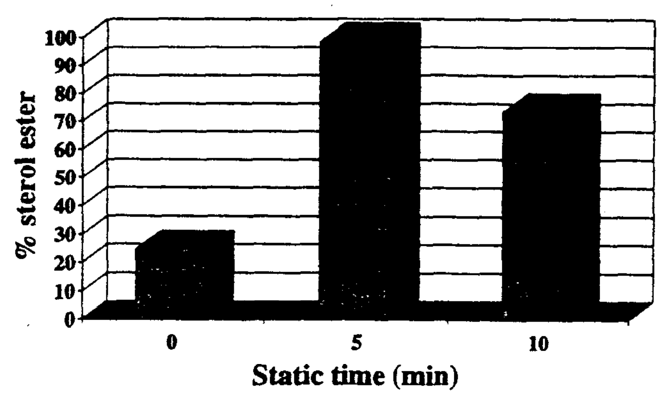

Fig. 5 Effect of static hold time on cholesterol palmitate yield. Conditions: 27.6 MPa, 50 °C, flow rate=1.0 ml/min

tial static hold period was found to aid in establishing solute (reactant) solubility in the SC-CO_2.

Similarly, the effect of reaction temperature on production of the cholesterol palmitate ester was also examined as shown in Fig. 3. The optimal reaction pressure of 27.6 MPa determined previously was used in these reactions as well as a CO_2 flow rate of 2.0 ml/min. Initial static hold times of 3 min were used before commencing the above syntheses. As indicated in Fig. 3, over the range of 40–60 °C it was found that a optimal ester yield of approximately 59% was achieved at 50 °C.

The effect of static hold time was also examined more thoroughly as a function of reaction pressure, temperature, and CO_2 flow rate. Figures 4 and 5 show two of the more promising results for the esterification between palmitic acid and cholesterol. As shown in Fig. 4, at 20.7 MPa, 40 °C, and a CO_2 flow rate of 2.0 ml/min, a yield of over 50% could be obtained using hold times of 5–10 min. At 27.6 MPa, 50 °C and a CO_2 flow rate of 1.0 ml/min (Fig. 5), a static hold time of 5 min was found to give a 98% yield, obviously a superior yield to that obtained at 20.7 MPa and 40 °C and doubling the CO_2 flow rate.

The results from these preliminary esterification reactions conducted in the semi-continuous mode were then applied to studying the reaction of fatty acids of varying chain length with both cholesterol and sitostanol. A reaction pressure of 27.6 MPa, temperature of 50 °C, and flow rate of 1.0 ml/min were used for these reactions. Static hold times of 5 min were used for all of the reported syntheses. These results are summarized in Fig. 6 for the even carbon number fatty acids from C_8 to C_{18}, the graph bar on the left representing the yield achieved with cholesterol, and the bar on the right showing the result attained when using sitostanol as a reactant.

Yields of over 90% were attained for all of the esters formed regardless of fatty acid chain length or identity of the sterol/stanol reactant. As shown in Fig. 6, there was a slight bias toward lower yields for both sterol/stanol moieties as the chain length of the fatty acid decreased; however, even for the C_8 ester, a yield of 90% and 92% were achieved with cholesterol and sitostanol, respectively. Reaction yields in excess of 98% were achieved in the case for the formation of the C_{10}, C_{12}, C_{16}, and C_{18} esters with cholesterol, while similar results were attained for formation of the sitostanol ester with C_{16} and C_{18} fatty acids. It should be noted that, in all cases for the sitostanol esters that were formed, the reaction yield was above 90%. This is important since it is these esters that are the reported functional ingredient in commercial products reported to lower cholesterol levels in animal and human subjects [16].

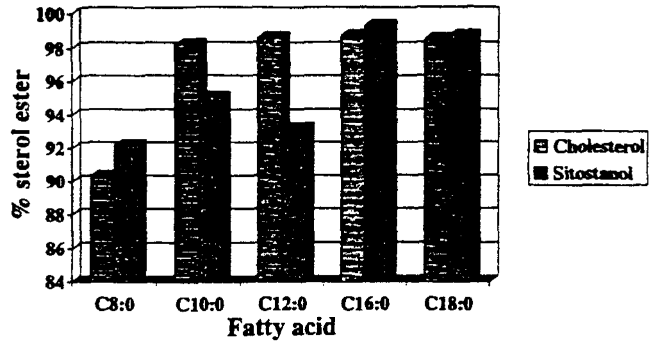

Fig. 6 Yields for various fatty acids reacting with cholesterol or sitostanol using Chirazyme L-1. Conditions: 27.6 MPa, 50 °C, flow rate=2.0 ml/min, static hold time=5 min

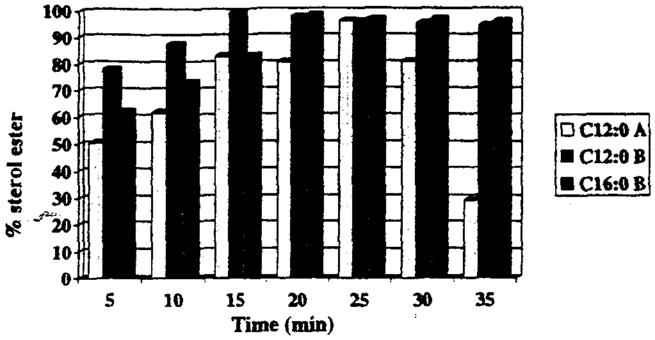

Fig. 7 Ester production in continuous flow system using Chirazyme L-1. Conditions: 27.6 MPa, 50 °C, A=cholesterol channel flow rate – 1.3 ml/min, fatty acid channel flow rate – 0.7 ml/min.; B=cholesterol channel flow rate – 0.9 ml/min, fatty acid channel flow rate – 1.0 ml/min

Similar reactions were also run between cholesterol and two fatty acids, C_{12} and C_{16}, using the continuous flow system described previously. These results are summarized in Fig. 7 for a reaction pressure and temperature of 27.6 MPa and 50 °C, at two different combinations of CO_2 flow rate passing through the reagent reservoirs containing either C_{12} or C_{16} fatty acids and cholesterol, respectively. For designated conditions A and B, these flow rates were for A: 1.3 ml/min for the cholesterol reservoir, 0.7 ml/min for the fatty acid reservoir; and for B, 0.9 ml/min for through the cholesterol reservoir and 1.0 ml/min through the reservoir containing the fatty acids.

As shown in Fig. 7, high reaction yields were attained in the flow system after about 15–20 min of run time. Depending on the particular combination of fatty acid and cholesterol and their respective flow rates of CO_2 through the reagent reservoirs, yields in excess of 80% were achieved in all cases. Yields of over 90% were recorded for the B conditions for cholesterol reacting with either the C_{12} or C_{16} fatty acids after 20 min of reaction time. No explanation can be given for the anomalously low yield found for the reaction between the C_{12} fatty acid and cholesterol under condition B after 35 min reaction time, other than that one of the reactants was depleted in its respective reservoir. However, these promising results suggest that a continuous flow synthesis of these esters is possible under the stated conditions.

In summary, a batch, semi-continuous method was developed for testing the feasibility of conducting esterifications between sterols and fatty acids in SC-CO_2. Various lipases were also evaluated with respect to their efficacy to catalyze the above reaction under the pressures and temperatures associated with SC-CO_2 extraction or reaction chemistry. Synthesis of alternative esters formed between various fatty acids and sitostanol was also evaluated and produced high yields. Finally, a continuous micro flow reactor, consisting of commercially-available pumps and components, was constructed and tested with respect to forming fatty acid esters of sterols or stanols. Ester yields of over 90% could be attained using this approach, suggesting a continuous method of producing nutriceutical-important esters for the functional food marketplace.

References

1. Jessop P, Leitner W (1999) Chemical synthesis using supercritical fluids. Wiley-VCH, Weinheim
2. Aaltonen O, Rantakyla M (1991) Chemtech 21:240–248
3. Gunnlaugsdottir H, Jaremo M, Sivik B (1998) J Supercrit Fluids 12:85–93
4. Overmeyer A, Schrader-Lippelt S, Kasche V, Brunner G (1999) Biotechnol Lett 21:65–69
5. Habulin M, Krmelj V, Knez Z (1996) J Agric Food Chem 44: 338–342
6. Mesiano A, Beckman E, Russell A (1999) Chem Rev 99: 623–633
7. Randolph T, Blanch H, Clark D (1991) Biocatalysis in supercritical fluids. In: Dordick J (ed) Biocatalysts for industry. Plenum, New York, pp 219–237
8. King J, Jackson M, Temelli F (1995) Development of industrially-useful synthetic processes in supercritical carbon dioxide. In: Kikic I, Alessi P (eds) I Fluidi Supercritici e le Lorno Applicazioni. Trieste, pp 19–26.
9. Jackson M, King J (1996) J Am Oil Chem Soc 73:353–356
10. Jackson M, King J (1997) J Am Oil Chem Soc 74:103–106
11. Jackson M, King J, List G, Neff W (1997) J Am Oil Chem Soc 74:635–639
12. Bornscheuer U, Kazlauskas R (1999) Hydrolases in organic synthesis: regio- and stereoselective biotransformations. Wiley-VCH, Weinheim
13. Snyder J, King J, Jackson M (1996) J Chromatogr 750:201–207
14. Snyder J, King J, Jackson M (1997) J Am Oil Chem Soc 74: 585–588
15. Andersson M, King J, Blomberg L (2000) Green Chem 2: 230–234
16. Moreau R, Norton R, Hicks, K (1999) INFORM 10:572–577
17. Miettinen T, Vanhanen H, Wester I (1996) Use of stanol fatty acid ester for reducing serum cholesterol. US Pat 5,502,045
18. Miettinen T, Gylling (1997) Sitostanol-ester margarine. In: Yalpani E (ed) New technologies for healthy foods and nutriceuticals. ATL Press, Shrewsbury, MA, USA, pp 71–83
19. Frykman H, Snyder J, King J (1998) J Am Oil Chem Soc 75:517–520